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13. ABSTRACT *(Maximum 200 Words)*

Virtually all human prostate cancers harbor a common somatically acquired genetic lesion - loss of expression of the carcinogen defense enzyme glutathione *S*-transferase-π (GST-π) due to methylation of deoxycytidine residues in the promoter region of the gene. We have hypothesized that this lesion, acquired early in prostate carcinogenesis, renders prostate cells susceptible to accumulating genetic damage and ultimately to frank carcinoma. We propose to investigate whether a suitable prostate cancer preventive strategy could involve up-regulation of global carcinogen defenses (phase 2 enzymes) by chemical or dietary means. Over the past year, we have made extraordinary progress in developing this hypothesis. We have identified sulforaphane, a dietary isothiocyanate found in cucifers, as the most potent phase 2 enzyme inducing agent in human prostate cancer cell lines compared to over 50 other compounds screened in our laboratory. Sulforaphane readily induced increased expression of quinone reductase, GST-α, and gamma-glutamylcysteine synthase, the rate limiting enzyme in glutathione synthetic pathways. These alterations are associated with increased enzymatic activity (QR and GST) and increased intracellular glutathione levels. We are completing a fascinating set of experiments characterizing global changes in mRNA expression for nearly 10,000 genes simultaneously using cDNA microarrays after treatment of prostate cells with sulforaphane. These experiments document that sulforaphane rather selectively induces a battery of phase 2 enzymes. From this work, we are confident that appropriate molecular biomarkers will be identified that will serve as strategic endpoints in future prostate cancer prevention trials.

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Prostate Cancer Prevention Through Induction of Phase 2 Enzymes

New Investigator Award

James D. Brooks, M.D.
Stanford University School of Medicine, Department of Urology

Introduction

In the earliest stages of its development, human prostate cancer loses expression of the enzyme glutathione *S*-transferase-π (GSTP1) a member of the class enzymes of carcinogen defense referred to as phase 2 enzymes. Since prostate cancer is crippled in one aspect of its carcinogen defenses, we have speculated that global induction of the class of phase 2 enzymes may hold promise as a prostate cancer prevention strategy. The subject of this research project is to identify whether induction of phase 2 enzymes (carcinogen defense enzymes may be a suitable tartegt for a prostate cancer prevention strategy. The purpose is to define the spectrum of phase 2 enzyme response in human prostate cancer cells *in vitro* so beneficial preventive targets may be evaluated *n vivo* in human prostate cancer preventive trials. This mechanistically based approach offers the advantage of immediately suggesting biomarkers of efficacy that can be measured in short term clinical trials – namely, measuring increased expression of carcinogen defense enzymes in the prostate after treatment with a putative preventive agent. Since prostate cancer is typically slow-growing requiring years to progress and prove fatal, short term strategic trials will be critical in developing suitable agents for testing in larger, long-term clinical trials. Through such trials, the ideal "phase 2 inducing" prostate cancer preventive agent could be identified and targeted for larger, more costly clinical trials of efficacy.

Body

Our first objective has been to identify a potential prostate cancer preventive agent based on its ability to induce increased phase 2 enzyme expression and activity. We have assessed over 50 candidate carcinogen detoxification enzyme inducer compounds for their ability to induce increased NADPH menadione oxidoreductase (quinone reductase or QR) activity *in vitro* in the human prostate cancer cell lines LNCaPazaC and LNCaP cells, which make and do not make GSTP1 respectively, and in human liver cells (HepG2) (1). The screening strategy involves quantitative detection of quinone reductase (QR) activity after exposure of prostate cancer cell lines to inducing agents (2). Quinone reductase is induced coordinately with other phase 2 enzymes and remains stably expressed in cell culture. Striking differences in induciblity of QR were seen between the prostate and liver cell lines. Since starting on this project funded by the USAMRMC, we have identified sulforaphane as one of the most potent phase 2 enzyme inducing compounds in the prostate cancer cell lines. Sulforaphane was first identified as a micronutrient found at high levels in cruciferous vegetables, has documented cancer preventive activity in animal models and has been implicated as a potential preventive agent in epidemiologic studies (3-5). Recently, broccoli sprouts have been found to be a rich source of sulforaphane and suitable for use in clinical trials (6). We have now focused much of our effort on this intriguing compound. Induction of QR enzymatic activity with sulforaphane at nanomolar concentrations was observed in several prostate cancer cell lines and appears to be transcriptionally mediated. In the prostate cancer cell line LNCaP, QR enzymatic activity increased 2-3 fold over DMSO treated control cells 48 hours after treatment with 10 μM sulforaphane. Transcriptional induction of QR, glutathione *S*-transferase-α and microsomal glutathione transferase was measurable by northern blot analysis in several human prostate cancer cell lines at 8 hours (Figure 1).

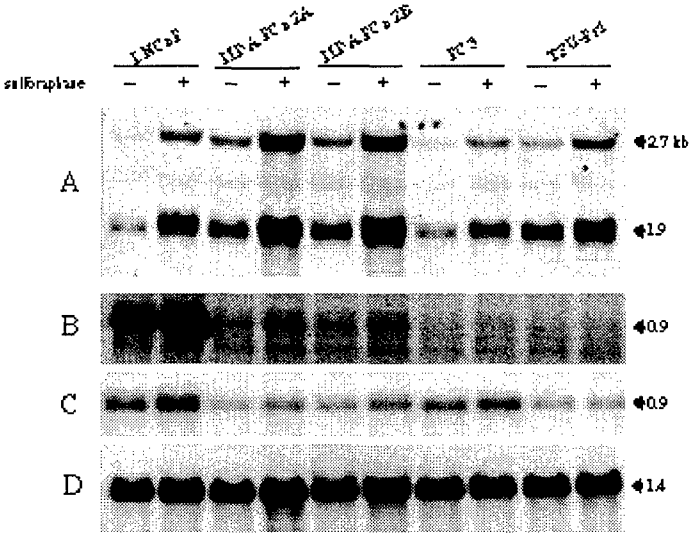

Figure 1: A. Induction of quinine reductase mRNA by 10 μM sulforaphane. Three transcripts are seen as has been reported previously. Note robust induction by sulforaphane. B. Glutathione *S*-transferase-α C. Microsomal glutathione transferase D. GAPDH probe of the same blot.

Time course experiments show transcriptional induction of QR within 4 hours of treatment, peaked by 8 hours at levels 3-4 fold above control, and persists for up to 96 hours after a single treatment *in vitro* (Figure 2). Concurring with this observed transcriptional induction, elevations of QR enzymatic activity persisted up to five days following treatment (data not shown). Sulforaphane treatment also produced a persistent elevation of gamma-glutamylcysteine synthase mRNA, the rate limiting step in glutathione synthesis (Figure 2).

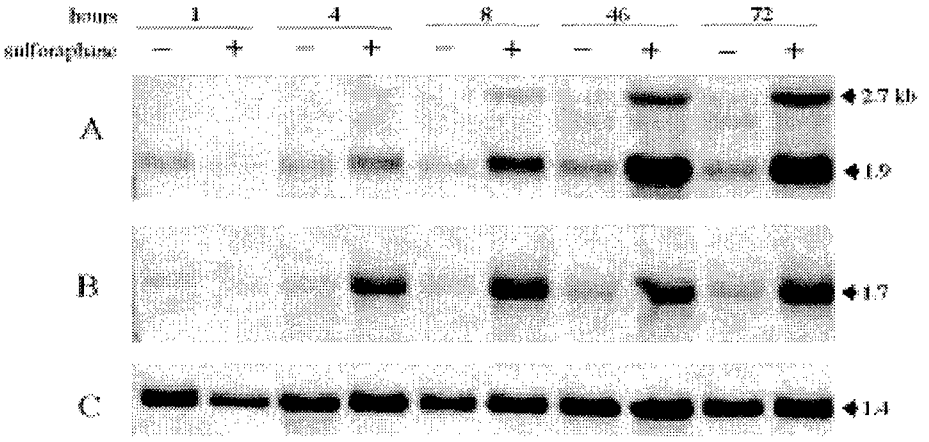

Figure 2 Time course of changes in gene expression after treatment with sulforaphane 10 μM as assessed by northern blot analysis. A. Quinone reductase expression with three transcripts as above. B. Gamma-glutamylcysteine synthase (light chain). C. GAPDH loading control probed on the same blot.

We have now confirmed these findings in the prostate cancer cell lines PC-3, DU-145 MDA-PCa 2A and MDA-PCa 2B as well as a prostate cancer epithelial cell strain obtained from Dr. Donna Peehl in our institution (Figure 1 and below). A 1.2-fold elevation of intracellular glutathione levels has also been documented after treatment with sulforaphane. This increased level of glutathione reflects induction of glutathione synthetic enzymes, specifically, the light chain of gamma-glutamylcysteine synthase.

Having identified an intriguing candidate phase 2 inducing agent for prostate cancer preventive approaches, we have now begun to develop an approach for identifying suitable biomarkers for measurement of efficacy. In our application, we had proposed utilizing one of the phase 2 enzymes like those mentioned above as "markers" of efficacy that could be measured in patients in clinical trials. We have had some concern, however, that the few biomarkers we can readily evaluate by northern blot analysis and/or enzymatic assay may not be useful as markers in clinical trials. This would leave us with the difficult question of deciding a whether a compound is simply ineffective at inducing phase 2 enzymes or it simply does not induce the single biomarker we are interrogating even though it may induce other phase 2 enzymes. Fortunately, the USAMRMC has given us the opportunity to develop a new technology in the laboratory that will circumvent these difficulties. We have begun to analyze global changes in gene expression after treatment of LNCaP cells with sulforaphane with cDNA microarray

technology. We are using an approach developed by Dr. Patrick Brown in the Department of Biochemistry at Stanford which allows measurement of differential gene expression between untreated and treated cells. This method has been exhaustively described in several recent publications (7-11).

My laboratory has become well versed in cDNA microarray technology as a direct result of funding from the USAMRMC. This technique, involving simultaneous assessment of expression of multiple genes, has been developed by Patrick O. Brown and David Botstein at Stanford. Methods are now well established to measure changes in gene expression for 20,000 genes with a single hybridization. A large core bioinformatics investigators are currently in place and have developed algorithms for interpretation and manipulation of the large datasets generated by these experiments (9). These computational systems have allowed extraction of biological insights from a number of microarray experiments in yeast and mammalian systems. Drs. Brown and Botstein have generously assisted in transferring this technology to my laboratory and remain close collaborators on a large related project assessing gene expression in prostate carcinomas recently funded by the National Cancer Institute.

The low cost, high sensitivity, accuracy and high throughput of cDNA microarrays is now well established (7-11). My laboratory has now manufactured and run experiments on a large number of arrays with easily interpretable and highly reproducible results. We have validated expression levels observed on arrays with northern blot analysis for several genes with a high degree of concordance.

We have recently assessed the effect of sulforaphane treatment on human prostate cells *in vitro* using cDNA microarrays of nearly 9000 genes and ESTs. To better characterize the effect of sulforaphane, we assessed its effect on global patterns of gene expression in the human prostate cancer cell line LNCaP. After treatment, poly-A RNA was extracted at 0, 2, 4, 8, 16 24, 48, 60 and 96 hrs and arrayed with mRNA from control LNCaP cells treated with vehicle alone and harvested at parallel time points. Several hundred genes were up or down regulated at least 2-fold after treatment with sulforaphane (not shown). Our initial set of experiments confirmed the extraordinary phase 2 enzyme inducing capabilities of sulforaphane (Figure 3).

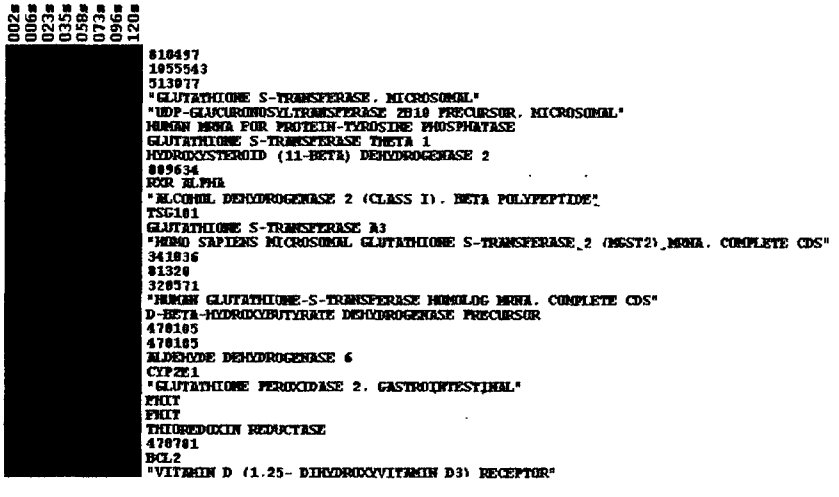

Figure 3: Gene expression induced by sulforaphane.

The microarrays corresponded precisely with our findings on northern blot analysis. Subsequent experiments have refined these observations with the inclusion of additional prostate cell lines and experiments in which LNCaP was treated with other phase 2 inducing compounds. Note prominent induction of phase 2 enzymes and thioredoxin reductase (Figure 4).

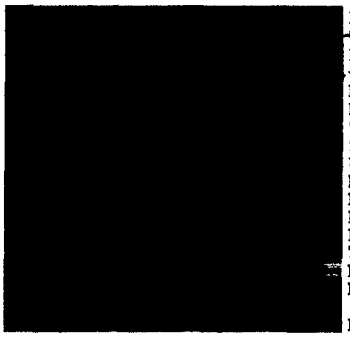

Figure 4: Genes up-regulated by treatment with sulforaphane. Columns 1-9 LNCaP timcourse, 10: MDA Pca 2A, 11: Normal Prostate epithelial cells, 12: PC3
Columns 13-16 LNCaP treated with other known Phase 2 enzyme inducers !#: Catechol, 14: Curcumin, 15: Dimethyl fumarate, 16: Hydroquinone.
Column 17: LNCaP ttreated with menadione (an -OH generator) and 18: Adriamycin
Degree of brightness corresponds to degree of increased expression compared to matched controls.

Equally interesting were the genes down-regulated by sulforaphane. In the subset shown in figure 5, a large cluster of metallothionines is appreciated. This finding was somewhat surprising since these genes are usually induced in response to cellular stress. Sulforaphane also appears to act by slowing proliferation (decreased PCNA) and decreasing growth factor receptor expression (Figure 5).

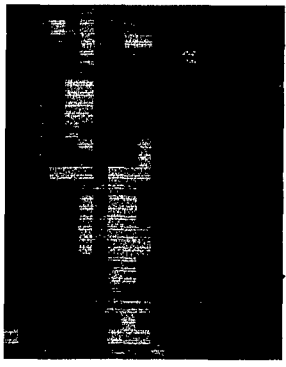

Figure 5: Genes down regulated by sulforaphane. Note the cluster of metallothionine genes down-regulated by sulforaphane. Growth factor receptors included in this figure (EGFR and Endothelin B receptor) are also down-regulated. Sulforaphane may decrease proliferation (PCNA) affect other signalling pathways. Genes displayed in this cluster demonstrate the richness of data which provide mechanistic insights to the action of nutritional agents. Degree of brightness corresponds to degree of decreased transcript copy number compared to matched controls. Columns as in Fig. 4

Recently, we evaluated expression patterns induced by an aqueous extract of broccoli sprouts, a known natural source of sulforaphane. We were delighted to observe that gene expression pattern changes closely matched those seen after treatment with sulforaphane (not shown). Thus, it is likely, that sulforaphane is the principal biologically active compound in broccoli sprouts and suggests that sprouts would be a suitable source of sulforaphane for use in clinical trials.

The above work satisfies the objectives outlined in my original work statement "To characterize phase 2 enzyme induction in human prostate cells *in vitro*." In many respects, our work far exceeds what we had outlined in the work statement. We had proposed to evaluate known phase 2 enzymes for induction by sulforaphane. While technically feasible, such an approach introduces bias in selection of which markers are assessed. Microarrays eliminate many of these biases by displaying the spectrum of response to a compound. Using cDNA microarrays, we have evaluated the compounds proposed in our original work statement. We have identified new potential biomarkers of response, the most promising being thioredoxin reductase. We have also identified some of the mechanisms by which phase 2 enzymes generrally and sulforaphane specifically may exert their chemopreventive effects.

We continue to work on our second goal: "To test whether induction of phase 2 enzymes will attenuate oxidative stress in prostate cancer cell lines *in vitro*." We have initiated a set of experiments in which we are attempting to induce oxidative stress in LNCaP by treatment with androgen. Generation of H_2O_2 and $^{\cdot}OH$ radical after treatment with androgen has been reported by Ripple et al. and may explain some of the promoting effect of androgen on prostate cancer (12, 13). Unfortunately, we are having some difficulty reproducing these findings. After three attempts, we have not been able to measure and oxidative burst with DCF after treatment of LNCaP with androgen. We can readily measure these effects with H_2O_2 in media, suggesting that the DCF is generating appropriate signal. We are now reassessing our experimental design and objectives as they relate to this aim. Our goal is to test whether a phase 2 enzyme inducing agent can dampen or block the effects of oxidative damage in prostate cancer cell lines. Androgen seemed an intriguing way of generating oxidative stress in prostate cancer cells, but has proved elusive in our hands. Recently, we have evaluated other means of generating oxidative stress. Menadione is known to generate intracellular $^{\cdot}OH$ radical by redox cyclingas a semiquinone and reacting with O_2 (14). Menadione appears to generate oxidative stress in the PC-3 human prostate cancer cell line. After treatment with 10 μM menadione, PC-3 generate a gene expression profile consistent with oxidative stress with induction of DNA damage repair enzymes, superoxide dismutase, glutathione peroxidase, and a number of other genes. Surprisingly, many phase 2 enzymes are not induced. We are now confirming that these changes in gene expression correlate with oxidative stress using DCF. If we can generate a strong signal of oxidative stress, we will use menadione to generate oxidative stress and test whether phase 2 enzyme induction can attenuate this stress.

We have not begun work on our third objective "To investigate the pharmacokinetics of phase 2 inducing agents in human prostate cancer grown in a xenograft model." Animal care approval has been obtained, and we are beginning to design these experiments and intend to carry them out in the second year of our proposal.

Key Research Accomplishments

A. Establishment of the methods of gene expression analysis using cDNA microarrays in my laboratory.
B. Using cDNA microarrays to demonstrate the spectrum of phase 2 enzyme gene response to putative prostate cancer preventive agents.
C. Identification of novel biomarkers (e.g. thioredoxine reductase) using cDNA microarrays that are suitable for evaluation in further preclinical studies and in clinical trials.
D. Elucidation of novel mechanisms through which putative phase 2 enzyme inducing agents may exert their anticancer effects (e.g. down-regulation of growth factor receptors) using cDNA microarrays.
E. Validation of cDNA microarray findings using northern blot analysis and enzyme assays.
F. Identification of the micronutrient sulforaphane as a potent phase 2 enzyme inducing agent in human prostate cancer cells *in vitro.*
G. Demonstration that broccoli sprouts, a reported natural source of sulforaphane, also induce the battery of carcinogen defense enzymes comparable to the pure compound. Thus broccoli sprouts may serve as a dietary source for sulforaphane.

Reportable Outcomes

A. Presentations

James D. Brooks and Vincent Paton: Potent Induction of Carcinogen Defense Enzymes with Sulforaphane, a Putative Prostate Cancer Chemopreventive Agent. Innovators in Urology, Oxford England, July 28-30, 1999.

James D. Brooks: Sulforaphane and Gene Expression in Prostate Cells. Strategies for Developing New Clinical Trials for Prostate Cancer Chemoprevention Workshop. National Cancer Institute, Baltimore, MD, August 8-9, 1999.

James D. Brooks: Nutrition and Gene Expression" CaPCURE Sixth Annual Scientific Retreat. Lake Tahoe, Nevada, October 17, 1999.

James D. Brooks: Defining the mechanisms of prostate cancer chemopreventive agents using cDNA expression arrays. 8th Prouts Neck meeting on Prostate Cancer, Prouts Neck, Maine, October 23, 1999.

B. Publications

James D. Brooks and Vincent Paton: Potent Induction of Carcinogen Defense Enzymes with Sulforaphane, a Putative Prostate Cancer Chemopreventive Agent. *Prostate Cancer and Prostatic Diseases*, In press, 1999.

James D. Brooks and William G. Nelson: "Prostate Cancer Chemoprevention" in Prostate Cancer in the 21st Century. Chung LWK, Isaacs WB and Simons J (Eds.) In Press, 1999.

C. Funding

National Cancer Institute, NIH: "A Cancer Taxonomy Based on Gene Expression Patterns." (PI: Patrick O. Brown), Co-Investigator: James D. Brooks. October, 1999-September 2004. Total direct costs: $1,664,908 (year 1).

Conclusions

The USAMRMC New Investigator Award in Prostate Cancer has been critical to my development as a new independent investigator. This award has allowed me protected time to develop novel techniques in my laboratory and establish collaborations that have ensured funding for several years to come. This funding has allowed me to develop a novel, untested strategy in prostate cancer prevention that holds promise. After only one year, we have identified a micronutrient, sulforaphane, which may be suitable for clinical trials in prostate cancer prevention. We have gone on to perform ground-breaking work defining the mechanism of sulforaphane action using cDNA microarrays. This work has been extremely well received in the scientific community and has fostered my development as a new investigator. Furthermore, the findings from our first year will fuel several years of investigation in my laboratory. Much of this work will entail the functional studies outlined in the original proposal directed at answering the questions "Does sulforaphane attenuate oxidative stress?" and "What are the pharmacokinetics of sulforaphane?" Favorable answers to these questions should lead to a clinical trial to evaluate sulforaphane's ability to induce the phase 2 response in human prostates.

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